SPECIFICATION AMENDMENTS

Please replace paragraph [0001] with the following rewritten paragraph:

--**[0001]** This application is a continuation-in-part U.S. Serial No. 09/500,747, filed 9 February 2000 and now U.S. patent 6,753,173 which claims benefit of Provisional Application 60/119,363, filed 9 February 1999. The present application further claims benefit of U.S. Provisional Applications 60/272,985 and 60/272,987, both filed 2 March 2001. Each of these documents is incorporated herein by reference.--

Please replace paragraph [0015] with the following amended paragraph:

[0015] PCT publication WO 98/49315, the contents of which are incorporated herein by reference, describes an approach for modifying the enzymatic activities included within modules of a PKS by maintaining the scaffolding intact but replacing catalytic domains with different catalytic domains. U.S. Serial No. 09/346,860 filed 2 July 1999 and now U.S. patent 6,221,641 and the corresponding PCT publication WO 00/01838, also filed on that date, and incorporated herein by reference describe alternative methods by altering the hypervariable region of the AT domains so as to alter the specificity for an extender unit and alteration of the KS domains to control stereochemistry. The present invention takes advantage of the approach of manipulating modules so that the catalytic activities of an entire module are placed in the appropriate sequence to construct a desired polyketide. The ability to utilize this approach depends on effecting an appropriate means for the module to incorporate a growing polyketide chain, which involves assuring that an appropriate linker region is included. Since the filing of the provisional application from which the present application claims priority, a related paper has been published by Ranganathan, A., *et al., Chem. & Biol.* (1999) 6:731-741. In this paper, intrapolypeptide linkages are fortuitously supplied to chimeric modules by including the KS region of the native downstream module in a chimera between the corresponding upstream module and the portions downstream of the KS domain in a heterologous module. Alternatively, the downstream module will include the ACP catalytic domain of the native upstream module fused to the remainder of a heterologous module upstream in the chimera.

Please replace paragraph [0085] with the following amended paragraph:

[0085] A preferred starter unit for such an assembly of modules is a diketide thioester either formed *in situ* by including a module which contains a loading domain to incorporate a starter unit along with an extender unit to attain this resultant, or the diketide may be synthesized independently and used as the substrate for the PKS. The synthesized diketide may be supplied as the thioester, such as the N-acylcysteamine thioesters. Preparation methods for these thioesters are described in the above-referenced U.S. Serial No. 09/346,860 filed 2 July 1999 and the corresponding PCT application, as well as U.S. Serial No. ~~(Atty. docket No. 30062-20032.00)~~ 09/492,733 filed 27 January 2000, now U.S. patent 6,492,562.

Please replace paragraph [0097] with the following amended paragraph:

[0097] While physical channeling is a necessary outcome of fundamental polyketide biosynthetic mechanisms (Donadio, et al., *Science* 1991, *252*, 675-679; Cortes, et al., *Nature* 1990, *348*, 176-178), the kinetic advantage, if any, of channeling intermediates between modules has not yet been resolved. To elucidate the issue of "kinetic channeling" (which is defined as physical channeling that results in a kinetic advantage--as measured by k_{cat} sover a diffusive loading mechanism in which the intermediate equilibrates in the bulk phase after release from the upstream active site and before loading in the downstream active site) in modular PKSs, two new assay systems-one to probe intrapolypeptide transfers and one to probe interpolypeptide transfers--were devised that would more accurately mimic the transfer of a substrate from the acyl carrier protein (ACP) of one module to the ketosynthase (KS) of the next. These assays are described in further detail in Example 7 below. In the first assay system, the loading didomain and module 1 of DEBS generated in situ the natural diketide intermediate ((2*S*,3*R*)-2-methyl-3-hydroxy-pentanoyl-S-ACP_1), which could then be transferred to alternative downstream modules in a bimodular PKS context (Figure 10B). By comparing the kinetic parameters of these hybrid bimodular systems to those for elongation of the same diketide that has been supplied exogenously to the isolated downstream module (Figure 10A), the kinetic benefit of channeling intermediates between covalently linked modules could be evaluated. A second assay system was developed using a chemoenzymatic method, through which alternative diketides were covalently attached to the phosphopantetheine

arms of an individually expressed donor ACP domain (Figure 10C). Here, the entire diketide-S-ACP adduct (**4a-d**) is a formal substrate for a recipient module, therefore allowing investigation of interpolypeptide channeling. (The linker sequence at the C-terminal end of the ACP as previously described, *see* Tsuji, et al., *Biochemistry* [[~~2001~~]] (2001) 40:2326-2331, was included in this construct.) By attaching different diketides to the same ACP, the steady-state kinetic parameters for diketide elongation by individual modules (each with a TE domain fused to its C terminus to facilitate turnover) could be measured. Both assay systems were used to compare the properties of modules 2, 5, and 6 of DEBS, three modules that perform the same chemistry with identical stereocontrol, albeit on very different substrates (Figure 4). The results of these studies are described in Example 7 below.

Please replace paragraph [0131] with the following amended paragraph:

[0131] Construction of Plasmids. The gene encoding ACP4(4) was amplified as an *NdeI-Eco*RI PCR fragment (523 bp) using the primers 5'-CCATATG**GTGGTCGACCGGCTCG**-3'and 5'-GAATTC**CTACAGGTCCTCTCCCCC**-3' (sequences complementary to DEBS shown in bold). The PCR product was cloned into pET28a (Novagen) to yield plasmid pNW8. Plasmid pST157 encodes a bimodular fusion between module 1 of DEBS1 and module 5 of DEBS3, with the thioesterase domain fused downstream of module 5 ("M1+M5+TE"). This fusion, which was engineered by taking advantage of the natural, conserved *Bsa*BI sites located at the start of the KS domains of modules 2 and 5, also includes the loading didomain of DEBS1. The "linker" sequence that covalently bridges the fused modules is the natural sequence between modules 1 and 2, as in DEBS1. The fusion junction between module 5 and the thioesterase domain is identical to that in plasmid pRSG46.[23] Similarly, plasmid pST92 encodes an "M1+M6+TE" bimodular fusion. Its construction, which is completely analogous to that of pST157, involves introduction of this bimodular PKS gene from pST96, Gokhale, et al., *Science* 1999, *284*, 482-485, as an *Nde*I-*Eco*RI into pET-21c (Novagen). The construction of genes encoding (5)M2+TE, (3)M3+TE, (5)M5+TE, and (5)M6+TE (pRSG64, pRSG64, pRSG46, and pRSG54, respectively) have been described previously, *id.*, as well as the construction of a gene encoding (5)M3+TE (pST132). *See* Tsuji, et al., *Biochemistry* [[~~2001~~]] (2001) 40:2326-2331.

Please replace paragraph [0143] with the following amended paragraph:

[0143] Construction and Expression of Individual ACPs. ACP4-(4) includes the entire DEBS ACP4 catalytic domain with its natural C-terminal linker. (The ACP linker is defined as the residues between the ACP consensus sequence and the C terminus of the polypeptide. *See* Tsuji, et al., *Biochemistry* [[~~2001~~]] (2001) 40:2326-2331). This gene was expressed as a 20.5 kDa N-terminally His_6-tagged protein to preserve the natural sequence of the C-terminal linker. ACP4(4) was purified by affinity chromatography on a nickel column followed by a hydrophobic phenyl sepharose column to yield approximately 10-15 mg/L culture of purified apoprotein.

Please replace paragraph [0145] with the following amended paragraph:

[0145] Qualitative Assays of Diketide Incorporation by Acyl-ACPs. The acyl-ACP4(4) adducts **4a-d** were incubated individually with (5)M2+TE, (5)M5+TE, and (5)M6+TE in the presence of saturating concentrations of ^{14}C-methylmalonyl CoA extender unit and NADPH. For a given acyl-ACP, the products from modules 2+TE, 5+TE, and 6+TE were expected to be identical (Figure 11), since the modules catalyze the same set of reactions with identical stereocontrol (albeit normally on very different natural substrates). Both **4a** and **4b** were accepted and extended by all three modules. Likewise, the corresponding NAC-thioesters **2a** and **2b** have been shown to be substrates for the three modules.[26] Remarkably, however, **4c** and **4d** were also substrates for the three modules, even though no turnover of the corresponding NAC thioesters **2c** and **2d** was detected in the case of any module, Wu, et al., *Am. Chem. Soc.* 2000, *122*, 4847-4852 (It should be noted that elongation of **4c** and **4d** by modules 5+TE and 6+TE yielded minor quantities of unreduced triketide lactones, indicating less efficient β-ketoreductase activity on these two anti-diketide substrates than on the two syn-diketide substrates.). Consistent with previous linker studies,[23,27] while all four acyl-ACP adducts were observed to be substrates for (5)M3+TE, no product formation was observed from the incubation of any of the acyl-ACP adducts with (3)M3+TE, even though **2a** and **2b** have previously been shown to be readily incorporated and elongated when presented to either module 3+TE derivative, Wu, et al., *Am. Chem. Soc.* 2000, *122*, 4847-4852; Tsuji, et al., *Biochemistry* [[~~2001~~]] (2001) 40:2326-2331. Thus, matched linker pairs appear to be capable of enhancing the efficiency with which otherwise poor substrates can be

channeled between modules. Conversely, mismatched linkers can present a major barrier to the channeling of otherwise acceptable substrates between modules. Control studies performed with **2a** and **5a** showed that the two compounds are approximately equivalent substrates for the same modules (data not shown). From the amount of product detected in these ACP-mediated reactions, the efficiency of the PKS-catalyzed reaction could be estimated. Under typical assay conditions (20μM **4** and 1μM (5)M5+TE), 70% of the acyl-ACP was converted into triketide lactone in 1 h. Two conclusions can be drawn from this result. First, acyl-ACPs are significantly superior substrates to acyl-NAC thioesters. (Typically, millimolar concentrations of the NAC thioester must be used to detect comparable amounts of product under otherwise similar assay conditions.) Second, the assay system described in Figure 11 allows for monitoring multiple turnovers of the enzyme. Indeed, as described below, in all cases the maximum rates of consumption of the acyl-ACP substrates were comparable to or higher than the maximum rates of consumption of their NAC thioester counterparts (see below). Therefore, the association of the donor ACP and the acceptor module must be transient, and the dissociation rate constant of the ACP from the module must be significantly faster than the slowest step in the module-catalyzed elongation sequence.

Please replace paragraph [0150] with the following amended paragraph:

[0150] The preference of **2a** over its enantiomer **2b** for all modules was especially intriguing in light of the fact that the natural substrates for modules 3 and 6 share more structural similarities to **2b** than to **2a**. One explanation for this discrepancy was that the NAC thioester-based assay system (Figure 10A) may not entirely represent the mechanism of acylation of a multimodular system. While NAC thioesters substrates must be loaded diffusively onto the KS of a module (Figure 10A), polyketide intermediates are channeled from the ACP of one module to the KS of the downstream module via covalent transfer. Substrate channeling in a multimodular system can occur either between two modules on the same polypeptide (e.g., between modules 1 and 2; Figure 10B), or between two modules on separate polypeptides (e.g., between modules 2 and 3; Figure 10C). Further evidence that protein-protein interactions may influence the substrate specificities of individual modules emerged from previous experiments suggesting, that inter-polypeptide linkers--defined as the highly variable regions outside the consensus sequences of the modules--are involved in mediating selective intermodular chain transfer, Gokhale, et al., *Science* 1999, *284*, 482-485;

Tsuji, et al., *Biochemistry* [[~~2001~~]] (2001) 40:2326-2331. To investigate the balance of protein-protein interactions and enzyme-substrate interactions in controlling polyketide chain elongation, two assay systems that take intermodular interactions into account were used in this study.